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DETERMINATION WHETHER THE CAUSAL AGENT FOR MUSSEL DIE-OFFS IN THE MISSISSIPPI RIVER IS OF CHEMICAL OR BIOLOGICAL ORIGIN

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DETERMINATION WHETHER THE CAUSAL AGENT FOR MUSSEL DIE-OFFS IN THE MISSISSIPPI RIVER IS OF CHEMICAL OR BIOLOGICAL ORIGIN

Final Report

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INTRODUCTION

According to Parmalee (1967), "A variety of species of freshwater mussels ... have inhabited the major rivers of Illinois since the end of the last glaciation, for at least 8,000 to 10,000 years." Mussel populations were and are important constituents of stream and river ecosystems. These filter-feeders remove organic detritus from the water column and convert the organic matter into usable biomass for the fish, raccoons, and muskrats which feed upon them. Mussels also provide a stable place of attachment for aquatic insect eggs and larvae in an often-shifting substrate.

Through time, man has discovered several uses for this natural resource. Prehistoric Indians used mussels as food and their shells for utensils, tools, and ornaments. Indians and settlers alike prized the occasional pearl mussels yielded. In the late 1800's, the accidental discovery of a pearl often set off a "pearl rush" which usually lasted until the mussel beds in that and nearby streams were depleted.

In 1887 John Boepple, a button maker by trade, immigrated to the United States from Germany and found mussel shells from midwestern streams and rivers were an excellent raw material for making buttons. By the summer of 1902 it was estimated 20,000 men were harvesting mussels from the Mississippi River alone (Knott 1980). Danglade (1914) considered the Illinois River the most productive mussel stream (per mile) in the country, and about 1910 there were more than 2,600 boats engaged in mussel fishing on the Illinois. Parmalee (1967) stated there were nearly 200 pearl button factories in the United States by 1912, and their annual sales amounted to more than $6 million. The industry flourished until the mid-1940's, when the development and production of plastic buttons caused the collapse of the pearl button industry.

By the late 1950's the Japanese had perfected a method of culturing pearls in marine oysters. Since that time, freshwater mussel shells have been harvested from streams and rivers of the Mississippi and Tennessee river valleys and exported to Japan for use in the cultured pearl industry. The freshwater shells are sawn into slices, then the slices are cubed, pressure ground, and polished into beads or "nuclei" which are surgically implanted into the mantle tissue of saltwater pearl oysters. The oysters coat the nuclei with layers of pearl nacre during a culture period of up to three years. An overview of the cultured pearl industry was presented by Ward (1985).

Unexplained die-offs of freshwater mussels occurred in the Mississippi River, including its waters in Illinois, starting in 1982 (UMRCC, 1983 and Figure 1). According to experienced observers (commercial shellers, district conservation biologists and law enforcement officers) the largest die-offs occurred in the summers of 1982 and 1985. No mussel die-offs were reported in the Illinois River, but die-offs were reported from the Tennessee and Ohio drainages in 1985 (Neves 1987a). There are dead beds of mussels in the Illinois River, including one by Banner marsh at river mile 139.7 which we sampled by diving 15 August 1987. The divers thought the mussels were alive because the shells were embedded in the bottom in a natural position and were heavy as though they contained live animals. When the shells were brought to the surface and opened however, they were packed with mud, which evidently deposited after the animals died. Die-offs may not have been reported because the densities of mussels are generally lower on the Illinois than on the Mississippi hence the density of floating meats would have been lower and less noticeable. Also, there are fewer shellers and shell buyers on the Illinois River, so a die-off or decline is less likely to be detected and reported.

Prior to 1985, shell buyers for the cultured pearl industry purchased only fresh shells -- those collected from live or "recently dead" mussels. However in 1985, buyers began taking "old dead" shells from mussels that had died one to two years earlier (Mr. D.E. Ballenger, Mississippi Valley Shell Company, personal communication, June 1986), probably necessitated by a shortage of higher-quality shells from living mussels due to the die-offs and overharvest. In 1986, shell buyers in Illinois reported 45-75% of the shells they purchased were relic or old dead shells resulting from the die-offs of 1982 through 1986 (Fritz, 1987). Fritz (1987) estimated 2/3rds of the Illinois shells he observed at shell camps in 1986 were from mussels that had died one to three years earlier.

In 1983, mortality estimates from quantitative sampling of formerly productive mussel beds where die-offs had been reported were as high as 30-40% in Mississippi River pools 10, 14 and 15 (UMRCC 1983, Blodgett and Sparks 1987, Figure 1). The frequency, magnitude, and extent of these unexplained die-off events led to a national workshop on mussel die-offs held 23-25 June 1986 in Davenport, Iowa (Neves, 1987a). Workshop papers reported the extent and severity of die-offs, as well as initial attempts to determine their causes. According to Bates (1987) numerous mussel die-offs had occurred previously, although documentation was often incomplete. Apparently no reported die-offs were as widespread as those in 1982-1986.

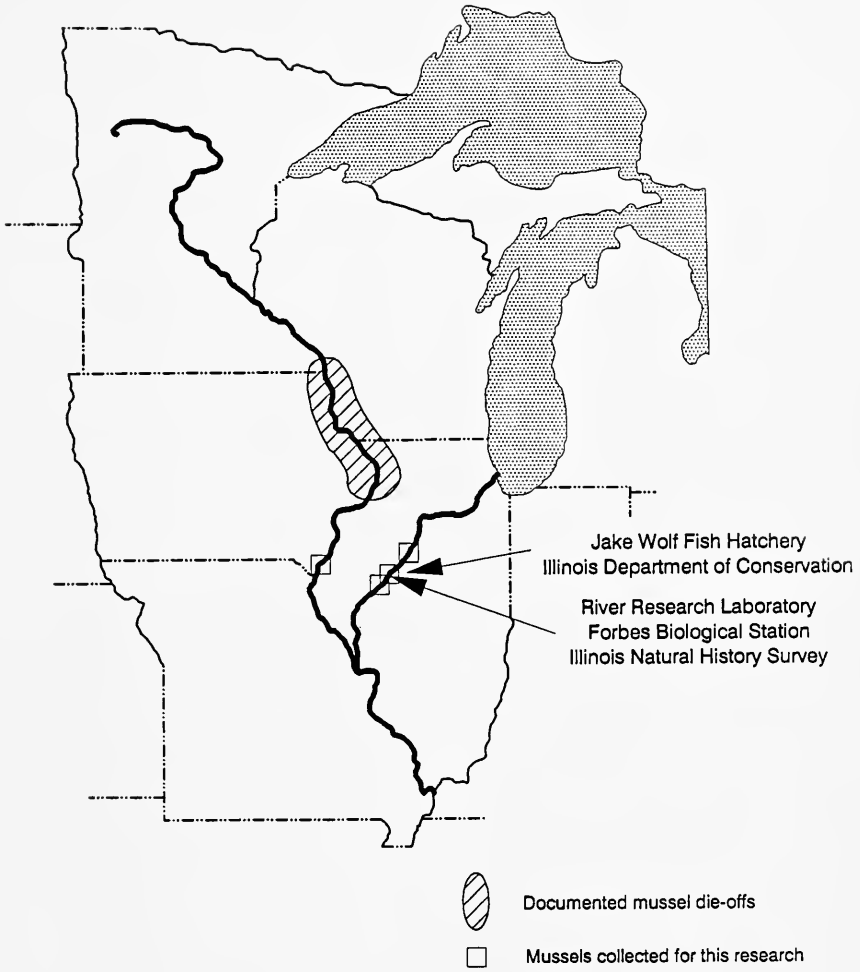

Figure 1. Locations of: (1) 1982-1986 mussel die-offs on the Upper Mississippi River, (2) sites where live mussels were collected for this study, and (3) the laboratories on the Illinois River where this study was conducted. Die-offs may have occurred as far south as the confluence of the Illinois and Mississippi rivers, according to observations by commercial shellers and fishermen; however, these observations were not verified by scientific sampling.

Published information on diseases of freshwater mussels is sparse. Only two freshwater mussel diseases were reported by Sparks in his review of invertebrate pathology (1985). He identified reports of tumor-like growths in freshwater mussels as early as 1890 and more recently by Pauley (1967a, 1967b, 1968). Two groups of parasites, trematodes and water mites, are known to infest freshwater mussels in the United States. Mites occur in mussels in our study reach of the Upper Mississippi River with the highest densities found in pink heelsplitters (*Potamilus alatus*) (Dr. Richard Anderson, Department of Biological Sciences, Western Illinois University, Macomb, Illinois 4 April 1990). The 1982-1986 die-offs occurred in many species, not just pink heelsplitters, so the mites themselves do not appear to be the cause. Vidrine (1980) indicated that the larvae of unionicolid water mites parasitize aquatic insects, while the nymphs and adults are symbionts with sponges, snails and mussels. Gale (1973) found that cercaria of the trematode parasite *Crepidostomum* (species not determined) killed most of the adult fingernail clams (*Musculium transversum*) at six of his sampling stations on Pool 19 of the Mississippi River in July and August 1967. Live, parasitized individuals were not gravid, so reproduction of the clams was reduced. However, mortality from trematodes and mites is usually rare in mussels (Neves 1987b). A small oligochaete worm, *Chaetogaster limnaei*, occurs in the Mississippi River in *Musculium transversum* (Gale 1973), Asiatic clams (*Corbicula fluminea*), and 5 species of mussels (Anderson and Holm 1987). Anderson and Holm (1987) and Gale (1973) did not find any evidence that *C. limnaei* harmed its hosts, and it is generally regarded as a commensal, not a parasite, of freshwater pulmonate snails (Brinkhurst and Jamieson 1971; Buse 1974). *Chaetogaster limnaei* might benefit mussels by consuming trematode miracidia and cercariae (Gruffydd 1965).

Havlik and Marking (1987) found an abundance of literature on uptake and storage of chemical contaminants by freshwater mussels, but little information is available documenting freshwater mussel mortalities attributable to contaminants in the environment (Neves 1987b).

Chemical and pathological analyses of dead and dying mussels collected during recent (1983-1986) die-off events failed to identify a causative agent (Ahlstedt and Jenkinson 1987, Jenkinson and Ahlstedt 1987, Thiel 1987, Scholla et al. 1987). However, some of these examinations uncovered potential clues which may facilitate the determination of the causative agent.

In 1983, Dr. Austin Farley, National Marine Fisheries Service Laboratory, Oxford, Maryland, examined living but moribund mussels collected from Illinois waters of the Mississippi River. He reported all specimens appeared to be in normal body condition, but they did have high densities of gram-positive, rod bacterium (Thiel 1987).

Scholla et al. (1987) examined bacterial flora and parasitic loads in both sick and apparently healthy mussels collected from the Tennessee River in 1985 and 1986. A gram-negative, rod bacteria which formed yellow colonies was present in both sick and healthy mussels, but sick mussels had significantly higher densities of the bacterium. When they incubated sick and healthy mussels together, the densities of the rod bacterium increased in the two initially healthy mussel specimens and neither responded to manual stimulation after a ten-day incubation, indicating a decrease in health possibly due to the bacteria.

Mr. Fred Kern, research biologist at the National Marine Fisheries Laboratory in Oxford, Maryland, performed histopathological examinations on live and decaying mussels collected from the Tennessee River during the die-off in 1985. Jenkinson and Ahlstedt (1987) reported the only major difference Kern found between live and decaying specimens was high levels of bacteria in the decomposing tissues of the latter. The presence of glycogen deposits adjacent to the digestive tract was interpreted as evidence the dead mussels had not starved to death.

Dr. Thomas Cheng, Medical University of South Carolina, conducted microbial and histological examinations on dead and dying mussels collected from the Tennessee River in January 1986. He concluded the specimens had been stressed by environmental factors which had "suppressed their immunological competence and allowed facultative invasion by bacteria" and that the immediate cause for death had been bacteriemia (Jenkinson and Ahlstedt 1987).

Mr. Rodney Horner, fish pathologist for the Illinois Department of Conservation (DOC), examined moribund mussels collected from Mississippi River Pool 12 in July 1985 by Mr. Bill Fritz, DOC commercial fisheries biologist. Horner reported the mussels were infected with various ciliated and flagellated protozoans and *Columnaris*-type bacteria. He also believed the mussels were invaded by the microorganisms after being stressed by some other factor. Horner reported that mussels from the Illinois River, where no die-offs had been reported, were relatively free of protozoans and bacteria (Thiel (1987). Mr. Howard Jackson, fish pathologist for the U.S. Fish and Wildlife Service Fish Disease Control Center in LaCrosse, Wisconsin, examined specimens from the same collection and he found no evidence of viral infection (Thiel 1987). However, it is possible the fish cell lines he used were inappropriate for investigating viral infections of mussels.

Results of examinations conducted by Mr. Kern on mussels collected from the Upper Mississippi River during the die-off in August 1985 were also reported by Thiel (1987). Kern found no evidence to support a diagnosis of mortality due to an infectious agent. He indicated specimens were generally in normal body condition and his diagnosis suggested "a sudden and acute onset caused by environmental conditions or toxins."

Because the mussels of Illinois are an important part of our aquatic ecosystems as well as an economically valuable resource, funds were provided by the Illinois Department of Energy and Natural Resources in November 1986 to investigate the cause of freshwater mussel die-offs in the Upper Mississippi River. The objectives of this project were to:

(1) determine whether the cause of the mussel die-offs in the Illinois waters of the Mississippi River was a biological or a chemical agent,

(2) characterize the normal commensal microflora of healthy mussels so that abnormal infections can be recognized, and determine whether abnormal infections precede morbidity or follow after the mussels are weakened by some other primary cause, and

(3) determine whether die-offs are triggered by an interaction between the agent and environmental stress, e.g. low oxygen concentrations, temperature extremes.

METHODS

In spring 1987 we constructed a 24- x 24-ft truss roof in the discharge basin of the Illinois Department of Conservation's Jake Wolf Memorial Fish Hatchery near Manito, IL (Figures 2 and 3). The roof provided shade and protection for three sets of duplicate 22-inch x 11-inch x 16-ft tanks (fiberglass troughs). The tanks were positioned on an incline and to each tank we added 2 to 3 inches of sand from the discharge basin for substrate.

Overflow water from the hatchery's conditioning pond flowed into a 125-gallon headbox via 4-inch pvc pipe. Water from the headbox was delivered via 4-inch pvc pipe to 6 independent 1-inch pvc feeder lines equipped with valves to allow us to regulate the amount of water dropped into the upstream end of each tank. Water flowed through the tanks and left the downstream ends via 4-inch pvc standpipes and was released into the sand discharge basin. Water levels in tanks were maintained approximately 6 inches above the substrate to facilitate visual inspections of the mussels. Prior to adding mussels each year, we maintained flow through the tanks for 3 weeks to leach solvents, plasticizers, and other potential contaminants from the system and to allow for establishment and stabilization of normal flora and fauna.

We collected mussels from the Illinois and Mississippi rivers by diving, brailing, and wading and transported them to the hatchery. The crowfoot bar, or mussel brail, is used by commercial shellers. It consists of multipronged hooks attached to a bar made from a pipe or a 2- x 4-inch board. A bridle and rope are attached to the bar, which is dragged over the mussel bed. When a hook enters the gape of a live mussel, the mussel clamps on the hook and is dragged from the bottom. After each river collection, a subsample of the mussels was delivered to the fish pathology laboratory for bacteriological analysis. These mussels were considered "fresh" mussels. We placed the remaining mussels into the tanks to be used as healthy stock for infectivity studies in the event of a die-off. When mussels in the tanks were subsampled later for bacteriological analysis they were classified as "cultured" mussels.

We assessed the relative health of each mussel prior to sampling for bacteria. Mussels were considered healthy if their valves closed rapidly and remained tightly closed when we touched the edge of the mantle. If a mussel was gaping and unresponsive to tactile stimulation (did not close its valves) over a prolonged period (1-3 hours) it was considered near death or dead and was classified as a mortality.

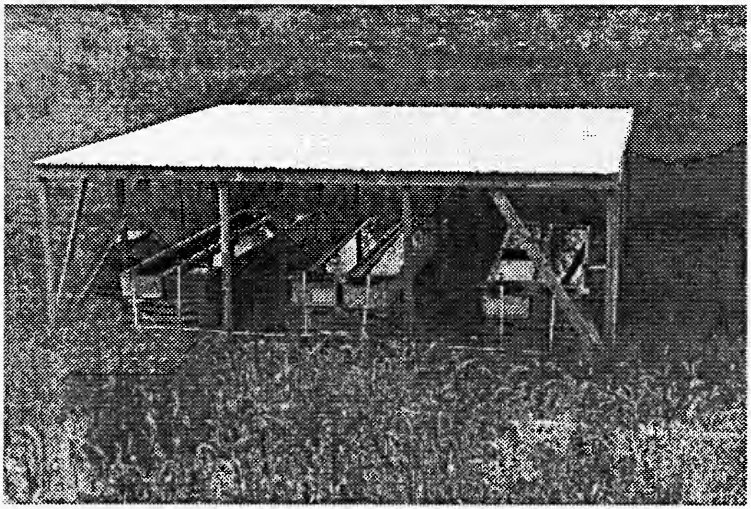

Figure 2. (Above) Tanks at the Jake Wolf Fish Hatchery where mussels were maintained for infectivity studies.

Figure 3. (Left) Closer view of mussel tanks.

Because a host of opportunistic decay microorganisms quickly invaded the dead mussels, bacterial isolations from mussels dead more than a few hours were of little value in this study. Therefore wet mounts (in deionized water) of gill tissue from mortalities were examined microscopically at 100x and 400x and only those with some beating gill cilia were sampled for bacteria. The presence of a foul odor was an immediate indication that the mussel had been dead too long for bacteriological sampling. We attempted to sample dead or apparently dying mussels as often as possible to provide a list of decay bacteria commonly associated with dead mussels. This list could be compared with the flora of moribund mussels collected during future unexplained die-off events to aid in recognizing uncommon species which may be causative agents for mortality. It is unlikely that decay organisms alone are responsible for massive mortalities of mussels--they are probably secondary invaders of mussels which have been stressed by other factors, including disease.

A standardized sampling method was developed to collect qualitative samples of bacterial species associated with various mussel tissues/organs including the stomach, mouth, hemolymph, midgut, gill, and mantle (Durham et al. in prep.). In general, bacteria were collected on sterile inoculating loops and plated onto brain heart infusion agar (BHIA) using the following methodologies.

In the laboratory, mussel shells were wiped with a paper towel that had been soaked in 95% ethyl alcohol. For healthy mussels, a sterile screw driver or oyster shucking knife was used to break the hinge ligament of the valves. A flamed inoculating loop was inserted into the opening in the stomach which was created near the anterior end when the hinge was broken. In weakened and dead mussels, the hinge did not need to be broken and the flamed inoculating loop was carefully inserted between the separated valves directly into the stomach. Stomach fluid and contents were plated onto BHIA. Next, excess fluid was drained, a sterile scalpel inserted to cut both adductor muscles, and a valve of the shell removed to reveal the body mass.

We collected hemolymph from the pericardial sack and heart. To prepare the pericardial sack for sampling, it was blotted dry with a Kimwipe or other tissue paper. To prevent reflooding of the sack and heart surface with fluid, it was sometimes necessary to press down on the foot of the mussel which in turn kept the area above the level of fluid in the shell. The surface was blotted with a second Kimwipe that had been soaked in 95% ethyl alcohol. Finally, the pericardial sack and heart was punctured with a sterile capillary tube which had been flame tapered to a small diameter at one end. Usually, clear hemolymph was collected in the tube, although not always with ease. We plated the hemolymph directly onto BHIA.

We prepared the midgut for sampling by first using a red hot scalpel blade to surface sterilize the proximal region of the mussel's foot. The same blade was immediately used to cut into the foot. A sterile inoculating loop was placed into the incision and the contents were plated onto BHIA.

To collect bacteria from gill tissue or ctenidium, the gill surface was disinfected using ethyl alcohol on a Kimwipe. We used a sterile scalpel to make an incision through one layer of the gill. An inoculating loop was carefully inserted into the gill and the contents plated onto BHIA.

The mouth and mantle were sampled infrequently. The mouth opening was sampled by inserting a sterile inoculating loop when the mouth was apparent. Fluid between the mantle and shell was collected by penetrating the mantle tissue with a red hot inoculating loop and plating the contents onto BHIA.

Inoculated BHIA plates were incubated at 30 degrees Centigrade for 24 hours. We isolated bacterial species in pure culture by repeated plating on BHIA. Identification of bacteria isolates was facilitated by standard bacteriological evaluations including Gram stain, cell morphology and motility, cytochrome oxidase production, O/F glucose medium, triple sugar iron agar, and the use of the MINITEK identification system for nonfermentors, Enterobacteriaceae II, and Gram-positive bacteria (BBL Microbiology Systems 1983a, 1983b, 1986). When preliminary tests indicated that 2 or more isolates from a single inoculation were of the same species, identification was attempted on only a single representative isolate. Therefore, data collected should be considered qualitative and not quantitative. In general, bacterial taxonomy follows that of *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt 1984).

RESULTS AND DISCUSSION

During this project over 600 freshwater mussels were collected from the Illinois and Mississippi rivers and transported to the Illinois Department of Conservation's Jake Wolf Memorial Fish Hatchery near Manito, Illinois. The tanks we constructed allowed us to establish and maintain healthy mussel stocks at the hatchery from July through December 1987 and May through November 1988 in anticipation of the infectivity experiments. Water flow through the tanks was sometimes reduced significantly for up to 3 days due to clogging of the delivery lines by filamentous algae from the conditioning pond. During these episodes, water temperature sometimes increased to over 30 degrees Centigrade and dissolved oxygen levels as low as 0.6 ppm were recorded. Although these episodes did not cause immediate, dramatic increases in mortality rates, they undoubtedly stressed the mussels and may have caused some mortalities over time.

Plans were made to collect dead and dying mussels from the Mississippi River during any unexplained die-off events and to attempt to infect the healthy specimens. However, there were no verified reports of mussel die-offs of unknown causes during this project, so the planned infectivity studies could not be undertaken.

A total of 93 mussels were sampled for bacteria. We isolated bacterial colonies from 80 of these mussels (Tables 1 and 2), and we identified 334 isolates representing 37 taxa and a minimum of 35 bacterial species (Table 3).

Twenty-six of the mussels from which isolates were identified were classified as mortalities (Table 2). In general, mortalities in the tanks were isolated events of 1-2 mussels dying at a time and not indicative of the massive simultaneous mortalities of 30-40% in the Mississippi River. Therefore, there is no strong evidence that any of the isolates we identified are likely to have been the sole agents responsible for the 1982-1986 die-offs in the Mississippi River.

We were unable to identify some bacterial isolates to species because of biochemical variabilities and disagreements on the taxonomy of some strains, inconclusive identification techniques, and time and manpower constraints. These isolates included various rod-shaped Gram-positive bacteria we classified only as "Gram positives", others we identified to Group VA-1 (Center for Disease Control Grouping), and finally those classified as *Alcaligenes* species, *Flavobacterium* species, *Moraxella* species, and *Pseudomonas* species (Table 3).

Of the 37 taxa we identified during this project, 7 were identified only in mussels from the Mississippi River and 15 found only in mussels from the Illinois (Table 3). Seven taxa were unique to healthy mussels and 10 unique to dead or dying specimens, although as mentioned above, most of the latter appear to be ubiquitous decay organisms which were taking advantage of a readily-available food supply.

We identified 23 bacterial species in samples taken from the hemolymph (Table 3) -- 18 from the Illinois River mussels (Table 4) and 14 from the Mississippi (Table 5). Sampling the hemolymph may be one of the best techniques to investigate for a systemic bacterial infection in mussels. This is based on the assumption that bacteria found in high numbers in the major circulatory organ constitute a septicemia. Obtaining a sample of hemolymph from the heart was often difficult and the potential for contamination from the digestive tube is appreciable because this tube passes directly through the heart. Additional contamination may result from the surface of the pericardial sac, as it is sometimes difficult to prevent fluids from coming into contact with the disinfected area during sampling.

Twenty-five bacterial taxa were identified in samples taken from the midgut (Table 3) -- 17 from the Illinois River and 15 from the Mississippi (Tables 4 and 5). Midgut samples were less likely to be contaminated than those from the pericardial sack because the flamed scalpel literally burned the surface of the foot where an incision was then made, and flooding of the area was not a problem. The stomach may provide an indication of environmental water quality. Bacteria in the water column are consumed by the filter-feeding mussels and may accumulate in the stomach.

All of the 12 bacterial species Cheng isolated and identified from dead and dying mussels collected from the Tennessee River during the die-off in 1986 (Jenkinson and Ahlstedt 1987) were found during this study with the exception of *Klebsiella oxytoca* (and possibly what they listed as *Pseudomonas fluorescens-putida*). Because Cheng described the extent of the infections of *Klebsiella oxytoca* as light and the species was reported from only 3 specimens (of at least 12 examined), it is doubtful that it could be considered the cause of that die-off. Species common to both studies were found in both Illinois and Mississippi River mussels, with the exceptions of *Vibrio alginolyticus* and *Klebsiella pneumoniae* which we identified only in mussels from the Illinois. Those species common to both studies were collected from both healthy and dead or dying specimens during our study with the exception of *Acinetobactor lwoffi* which we found only in healthy mussels.

The occurrence of these bacteria in healthy mussels indicates they probably are not the agents responsible for the die-offs in the Mississippi. Virulent pathogens of mussels would be expected to cause high mortalities, especially when low oxygen levels and high temperatures weakened our mussels and made them more susceptible to infection.

The gills and other soft tissues of mussels are bathed in warm river water rich in organic matter and microbes. It is likely that mussels have evolved bacteriostatic or bacteriocidal mucus, immunological responses, and other defenses against pathogenic, saprophytic, and fouling microorganisms, so the presence of bacteria in or on the animal in the absence of any symptoms is not an indication of disease. Indeed, some bacteria may be symbionts. Lautie et al. (1988) suggested that a bacterial symbiont in the freshwater mussel *Anondonta cygnea* was coupling the oxidation of manganese with the reduction of iron as an energy source and forming manganese concretions in the interstitial spaces of the connective tissue of the mussel. Some marine bivalves, such as *Solemya velum*, use their hemoglobin to transport reduced sulfide to intracellular, sulfide oxidizing, chemoautotrophic bacteria (Doeller et al. 1988).

Some of the species of bacteria we collected from mussels are known to cause disease in other aquatic organisms. For example *Aeromonas hydrophila*, the bacterium most frequently identified (Table 3), is a common waterborne saprophyte which can be isolated from nearly any freshwater source, and while *A. hydrophila* can cause mortality in aquatic organisms, it is generally considered a secondary invader associated with stressed organisms (Post 1987). We frequently found *Plesiomonas shigelloides* and *Vibrio fluvialis* in association with *A. hydrophila* in healthy as well as dead or apparently dying specimens. We did not attempt to quantify bacterial loads of the mussels sampled, but it was apparent that dead or dying mussels had higher levels of bacteria on the initial inoculations. Similar observations were reported by Scholla et al. (1987), Jenkinson and Ahlstedt (1987), and Thiel (1987). We identified *Vibrio alginolyticus* in 5 of our cultured mussels. It has been linked to juvenile abalone vibriosis -- a disease which causes mortality in a marine mollusk, the cultured red abalone, *Haliotis rufescens* (Elston and Lockwood 1983). Very little work has been done on pathogens of freshwater mussels and it would be worthwhile to determine whether *V. alginolyticus* affects freshwater mussels.

The absence of a die-off in the Upper Mississippi River during this study indicates either that the causative factor is now gone from the river or that the surviving mussels are resistant to it. If the cause was an infectious agent, it is possible that the survivors have developed antibodies which could be detected by biochemical tests. If the cause was a toxic chemical, the survivors may show greater resistance to the toxicant than mussels from an unexposed population.

The resistance hypothesis should be tested by exposing mussels from a river where there have been no die-offs to mussels, sediments, and water from the Upper Mississippi River. If the Mississippi mussels harbor an infectious agent to which they are resistant, the susceptible mussels from the unexposed population should catch the infection and suffer heavy mortalities. Such infectivity tests should be done in a laboratory where there would be no risk of introducing disease into a previously uninfected stream. The mussels should be maintained in unpolluted sediments and water during the infectivity tests. The clean water and sediments then should be replaced with material from the Upper Mississippi River, to test for toxic chemicals. If toxicants are present, the mussels from the Upper Mississippi River probably would be more resistant than the mussels from the river where no die-offs had occurred. These experiments could be done easily in a laboratory next to the Mississippi River, where fresh sediment could be obtained and water could be pumped directly from the river. Another approach would be to transfer mussels between the Upper Mississippi River and a reference river. If mussels from the reference river die in the Mississippi, while the Mississippi mussels thrive in the reference river, we would conclude that the Mississippi harbors an agent harmful to mussels. The disadvantage of the latter approach is that it risks introduction of an infectious agent into a previously unexposed population; hence the 2-step laboratory approach is preferred.

If the infectivity and antibody tests are negative (no biological agents) and the toxicity tests with raw water and sediment are positive (chemical toxicants present), standard toxicity identification and evaluation procedures (TIE) should be followed (Mount and Anderson-Carnahan 1988, Mount and Anderson-Carnahan 1989, Mount 1988, Ankley et al. 1989).

If chemical toxicants are no longer present, the mussels may still indicate the identity of the toxic agent responsible for the 1982-1986 die-offs by their differential resistance to suspect toxicants. If mussels from the Mississippi are now more resistant to certain toxicants than mussels from a river where no die-offs occurred, then these toxicants probably are the ones responsible for the die-offs. This evidence from comparison of mussels from different locations can be confirmed by comparison of mussels from the same location, but of different ages. Some of the mussels which occur in the Mississippi can live for 50 years, so the record of an episode of toxicity (and elimination of sensitive individuals) can be preserved for a considerable time. All that is required is to compare the resistance of individuals alive at the time of the die-off to individuals spawned after the die-off. If the toxicant is now gone from the river, there is no longer any selection for resistance, and mussels spawned since 1986 gradually may become more sensitive than their older conspecifics. The younger the mussel, the less likely there is to be any inherited resistance. Mussels lay down annual rings on their shells, so aging the mussels should not be a problem.

We plan to continue to maintain healthy mussel stocks in the tanks at the hatchery near Havana so that infectivity tests can be attempted in the event of future unexplained mussel die-offs. As time and resources permit, we will continue to sample, isolate, and identify bacteria from healthy and dead or dying mussels from the Illinois and Mississippi rivers, as well as other bodies of water. The tolerance of the mussels for low oxygen and high temperatures will be assessed in the tanks. Finally, we will seek funding and facilities suitable for the resistance tests described above.

SUMMARY

We constructed a flow-through tank system which allowed us to maintain healthy mussels from the Illinois and Mississippi rivers. We were unable to conduct infectivity experiments to determine whether or not the cause for the massive mussel die-offs in the Upper Mississippi River from 1982-1986 was of biological origin, because the die-offs apparently ceased before our research began in 1987.

We developed sampling methodologies to collect bacteria associated with various mussel organs and tissues. These techniques were used to characterize the bacterial flora associated with mussels collected from the Mississippi River 1-2 years after the reported die-offs and from the Illinois River, where no die-offs had been reported. This characterization of the bacterial flora of freshwater mussels provides a baseline for comparisons should mussel die-offs resume in the future. Of the 37 types of bacteria identified, 7 were collected only from mussels from the Mississippi River and 15 only from the Illinois. Seven were found only in healthy mussels and 10 only in the few dead or dying individuals from the tanks. The latter types of bacteria did not spread or were not pathogenic because we never had a massive die-off of mussels in the tanks.

Although several species of bacteria we found are known to cause disease in other aquatic organisms, these bacteria were found in both healthy and dead or dying mussels, so they could not be classed definitively as pathogens of freshwater mussels. Dead and dying mussels contained greater populations of bacteria than healthy ones, but the microbes probably were not the cause of the morbidity but were most likely taking advantage of animals with immune systems weakened by other factors. We found *Vibrio alginolyticus* in 5 mussels cultured in our tanks. *Vibrio alginolyticus* is pathogenic to a marine mollusk, the red abalone, *Haliotis rufescens*, and its effect on freshwater mussels should be investigated.

It is possible that the surviving mussels in the Upper Mississippi River are resistant to whatever caused the die-offs. The resistance hypothesis should be evaluated by: (1) antibody and infectivity tests using the pathogens we have identified and mussels from both the Upper Mississippi River and a river where no die-offs have occurred, and 2) toxicity tests with sediments and water from the Upper Mississippi River. If no infectious agent is present, but water or sediments from the Mississippi are toxic to mussels from rivers where no die-offs occurred, the toxicants might be identified using standard toxicity identification and evaluation (TIE) procedures. If water and sediments are not toxic now, the resistance of mussels which survived the die-offs should be compared to younger mussels and to mussels from rivers where no die-offs occurred, for a suite of chemicals likely to have occurred at toxic concentrations in the Upper Mississippi River in 1982-1986. Any toxicant for which the survivors now have greater resistance than the unexposed mussels would be suspected of causing the original die-offs.

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Table 1. Key to abbreviations used in Tables 2-5.

Source

frsh	fresh from the river
cult	cultured (held in a hatchery raceway)

Relative health

hlth	healthy - responsive
mort	mortality - dead or apparently dying

Mussel species

AmPl	*Amblema plicata*
AnGr	*Anodonta grandis*
FuFl	*Fusconaia flava*
LeFr	*Leptodea fragilis*
PoAl	*Potamilus alatus*
PoOh	*Potamilus ohioensis*
QuQu	*Quadrula quadrula*

Tissue/Organ

gill	gill
hemo	hemolymph
mgut	midgut
mntl	mantle
mout	mouth
stom	stomach

Bacterial taxa

Group VA-1 (CDC grouping)	Temporary grouping by the Center for Disease Control

Table 2. Freshwater mussels from which bacteria were collected, isolated, and identified.

	Illinois River				Mississippi River				Total			
	frsh	cult	hlth	mort	frsh	cult	hlth	mort	frsh	cult	hlth	mort
AmPl	8	10	12	6	5	1	5	1	13	11	17	7
AnGr	0	1	0	1	4	1	4	1	4	2	4	2
FuFl	0	1	0	1	0	0	0	0	0	1	0	1
LeFr	4	3	5	2	2	1	2	1	6	4	7	3
PoAl	0	2	0	2	2	0	2	0	2	2	2	2
PoOh	4	10	7	7	3	0	3	0	7	10	10	7
QuQu	6	5	9	2	5	2	5	2	11	7	14	4
Total	22	32	33	21	21	5	21	5	43	37	54	26
Species	4	7	4	7	6	4	6	4	6	7	6	7

Note: See Table 1 for a key to abbreviations.

Table 3. Bacterial taxa collected, isolated, and identified during qualitative sampling of mussels from the Illinois and Mississippi rivers.

Bacterial taxa	River		Source		Tissue/Organ						Status		Mussel species							Total Number Bacterial isolates identified
	Ill.	Miss.	frsh	cult	stom	hemo	mgut	mntl	gill	mout	hlth	mort	AmPl	AnGr	FuFl	LeFr	PoAl	PoOh	QuQu	
Acinetobacter lwoffi	1	5	6	0	1	2	1	2	0	0	6	0	1	0	0	3	0	1	1	6
Aeromonas hydrophila	73	21	43	51	31	26	18	10	7	2	55	39	21	6	3	11	6	29	18	94
Alcaligenes species	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1
Citrobacter freundii	8	10	10	8	7	4	7	0	0	0	13	5	1	1	0	1	5	3	7	18
Enterobacter agglomerans	3	4	5	2	1	4	2	0	0	0	6	1	0	0	0	0	3	1	3	7
Enterobacter cloacae	5	8	3	10	6	6	1	0	0	0	3	10	2	5	0	3	0	1	2	13
Enterobacter vulneras	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
Flavobacterium meningosepticum	4	0	1	3	2	2	0	0	0	0	2	2	0	0	0	1	0	3	0	4
Flavobacterium species	2	0	0	2	1	0	1	0	0	0	0	2	0	0	0	0	0	1	1	2
Gram-positive	42	14	42	14	16	5	17	14	4	0	48	8	21	1	0	8	1	10	15	56
Group VA-1 (CDC grouping)	2	0	2	0	0	1	1	0	0	0	0	2	0	0	0	2	0	0	0	2
Hafnia alvei	6	3	3	6	5	3	1	0	0	0	4	5	6	1	0	0	0	1	1	9
Klebsiella ozonae	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	0	1	1	2
Klebsiella pneumoniae	3	0	1	2	1	0	1	1	0	0	1	2	0	0	0	0	0	2	1	3
Micrococcus luteus	6	10	14	2	5	6	5	0	0	0	13	3	7	1	0	3	0	2	3	16
Moraxella species	1	0	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
Pasturella species	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Plesiomonas shigelloides	19	3	5	17	11	4	4	1	2	0	10	12	8	2	0	1	0	6	5	22
Proteus mirabilis	0	1	0	1	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1
Pseudomonas aeruginosa	0	3	3	0	1	1	1	0	0	0	3	0	1	0	0	0	0	2	0	3
Pseudomonas cepacia	1	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Pseudomonas maltophila	0	3	3	0	1	2	0	0	0	0	3	0	1	0	0	1	0	0	1	3

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Note: See Table 1 for a key to abbreviations.

Table 3. (continued)

Bacterial taxa	River		Source		Tissue/Organ						Status		Mussel species							Total Number Bacterial isolates identified
	Ill.	Miss.	frsh	cult	stom	hemo	mgut	mntl	gill	mout	hlth	mort	AmPl	AnGr	FuFl	LeFr	PoAl	PoOh	QuQu	
Pseudomonas putida	6	3	8	1	2	4	3	0	0	0	7	2	1	1	0	6	0	0	1	9
Pseudomonas putrifaciens	15	0	13	2	8	2	4	1	0	0	13	2	4	0	0	4	0	1	6	15
Pseudomonas species	1	4	4	1	2	0	1	1	1	0	4	1	3	0	0	0	0	1	1	5
Pseudomonas vesicularis	2	0	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	2
Salmonella arizonae	2	0	0	2	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	2
Salmonella enteritidis	0	1	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1
Serratia liquifaciens	0	3	2	1	1	2	0	0	0	0	2	1	1	0	0	0	0	0	2	3
Serratia plymuthica	0	2	0	2	2	0	0	0	0	0	0	2	2	0	0	0	0	0	0	2
Streptococcus acidominimus	2	0	2	0	1	1	0	0	0	0	2	0	0	0	0	0	0	0	2	2
Staphylococcus cohnii	1	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1
Staphylococcus epidermitis	1	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1
Vibrio alginolyticus	5	0	0	5	3	0	0	1	0	1	1	4	4	0	0	0	1	0	0	5
Vibrio cholerae	1	1	0	2	0	1	1	0	0	0	0	2	2	0	0	0	0	0	0	2
Vibrio fluvalis	10	1	4	7	5	2	2	1	1	0	5	6	1	1	1	4	0	4	0	11
Vibrio parahemolyticus	2	5	4	3	5	2	0	0	0	0	4	3	1	1	0	1	2	1	1	7
Bacterial isolates identified	227	107	184	150	123	83	78	32	15	3	211	123	91	21	4	53	18	73	74	334
Total bacterial taxa	30	22	27	28	28	23	25	9	5	2	27	30	22	11	2	18	6	21	21	37

Note: See Table 1 for a key to abbreviations.

Table 4. Bacterial taxa collected, isolated, and identified during qualitative sampling of mussels from the Illinois River.

Bacterial taxa	Source		Tissue/Organ						Status		Mussel species							Total Number Bacterial isolates identified
	frsh	cult	stom	hemo	mgut	mntl	gill	mout	hlth	mort	AmPl	AnGr	FuFl	LeFr	PoAl	PoOh	QuQu	
Acinetobacter lwoffi	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	1
Aeromonas hydrophila	27	46	21	22	12	9	7	2	39	34	18	0	3	11	4	26	11	73
Citrobacter freundii	2	6	3	1	4	0	0	0	5	3	1	0	0	1	0	3	3	8
Enterobacter agglomerans	1	2	0	2	1	0	0	0	2	1	0	0	0	0	0	1	2	3
Enterobacter cloacae	1	4	4	1	0	0	0	0	1	4	2	2	0	0	0	0	1	5
Enterobacter vulneras	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1
Flavobacterium meningosepticum	1	3	2	2	0	0	0	0	2	2	0	0	0	1	0	3	0	4
Flavobacterium species	0	2	1	0	1	0	0	0	0	2	0	0	0	0	0	1	1	2
Gram-positive	29	13	13	3	11	11	4	0	35	7	16	0	0	6	1	7	12	42
Group VA-1 (CDC grouping)	2	0	0	1	1	0	0	0	0	2	0	0	0	2	0	0	0	2
Hafnia alvei	0	6	3	2	1	0	0	0	1	5	5	0	0	0	0	1	0	6
Klebsiella ozonae	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1
Klebsiella pneumoniae	1	2	1	0	1	1	0	0	1	2	0	0	0	0	0	2	1	3
Micrococcus luteus	5	1	2	4	0	0	0	0	4	2	2	0	0	3	0	0	1	6
Moraxella species	1	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	1
Pasturella species	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Plesiomonas shigelloides	3	16	9	4	3	1	2	0	8	11	7	2	0	1	0	6	3	19
Pseudomonas cepacia	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Pseudomonas putida	6	0	2	3	1	0	0	0	5	1	1	0	0	5	0	0	0	6
Pseudomonas putrifaciens	13	2	8	2	4	1	0	0	13	2	4	0	0	4	0	1	6	15

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Note: See Table 1 for a key to abbreviations.

Table 4. (continued)

Bacterial taxa	Source		Tissue/Organ						Status		Mussel species							Total Number Bacterial isolates identified
	frsh	cult	stom	hemo	mgut	mntl	gill	mout	hlth	mort	AmPl	AnGr	FuFl	LeFr	PoAl	PoOh	QuQu	
Pseudomonas species	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1
Pseudomonas vesicularis	1	1	1	0	1	0	0	0	1	1	0	0	0	1	0	1	0	2
Salmonella ariaonae	0	2	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	2
Streptococcus acidominimus	2	0	1	1	0	0	0	0	2	0	0	0	0	0	0	0	2	2
Staphylococcus cohnii	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1
Staphylococcus epidermitist	1	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	1
Vibrio alginolyticus	0	5	3	0	0	1	0	1	1	4	4	0	0	0	1	0	0	5
Vibrio cholerae	0	1	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Vibrio fluvialis	3	7	5	2	1	1	1	0	4	6	1	0	1	4	0	4	0	10
Vibrio parahemolyticus	0	2	1	1	0	0	0	0	0	2	0	0	0	0	0	1	1	2
Bacterial isolates identified	101	126	84	54	46	25	14	3	128	99	64	4	4	42	6	59	48	227
Total bacterial taxa	19	23	21	18	17	7	7	2	20	26	14	2	2	14	3	15	16	30

Note: See Table 1 for a key to abbreviations.

Table 5. Bacterial taxa collected, isolated, and identified during qualitative sampling of mussels from the Mississippi River.

Bacterial taxa	Source		Tissue/Organ				Status		Mussel species						Total Number Bacterial isolates identified
	frsh	cult	stom	hemo	mgut	mntl	hlth	mort	AmPl	AnGr	LeFr	PoAl	PoOh	QuQu	
Acinetobacter lwoffi	5	0	1	2	0	2	5	0	1	0	2	0	1	1	5
Aeromonas hydrophila	16	5	10	4	6	1	16	5	3	6	0	2	3	7	21
Alcaligenes species	0	1	0	0	1	0	0	1	0	0	1	0	0	0	1
Citrobacter freundii	8	2	4	3	3	0	8	2	0	1	0	5	0	4	10
Enterobacter agglomerans	4	0	1	2	1	0	4	0	0	0	0	3	0	1	4
Enterobacter cloacae	2	6	2	5	1	0	2	6	0	3	3	0	1	1	8
Gram-positive	13	1	3	2	6	3	13	1	5	1	2	0	3	3	14
Hafnia alvei	3	0	2	1	0	0	3	0	1	1	0	0	0	1	3
Klebsiella ozonae	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1
Micrococcus luteus	9	1	3	2	5	0	9	1	5	1	0	0	2	2	10
Plesiomonas shigelloides	2	1	2	0	1	0	2	1	1	0	0	0	0	2	3
Proteus mirabilis	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1
Pseudomonas aeruginosa	3	0	1	1	1	0	3	0	1	0	0	0	2	0	3
Pseudomonas maltophila	3	0	1	2	0	0	3	0	1	0	1	0	0	1	3
Pseudomonas putida	2	1	0	1	2	0	2	1	0	1	1	0	0	1	3
Pseudomonas species	4	0	2	0	1	1	4	0	3	0	0	0	1	0	4
Salmonella enteritidis	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1
Serratia liquifaciens	2	1	1	2	0	0	2	1	1	0	0	0	0	2	3
Serratia plymuthica	0	2	2	0	0	0	0	2	2	0	0	0	0	0	2
Vibrio cholerae	0	1	0	0	1	0	0	1	1	0	0	0	0	0	1
Vibrio fluvialis	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1
Vibrio parahemolyticus	4	1	4	1	0	0	4	1	1	1	1	2	0	0	5
Bacterial isolates identified	83	24	39	29	32	7	83	24	27	17	11	12	14	26	107
Total bacterial taxa	18	13	15	14	15	4	18	13	14	10	7	4	8	12	22

Note: See Table 1 for a key to abbreviations.

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16. Abstract (Limit: 200 words)

Unexplained die-offs of freshwater mussels have occurred in the Mississippi River, including the water of Illinois, from 1982 to 1986. This research was directed at determining the cause, biological or chemical, of these massive die-offs. During this project, over 600 freshwater mussels were collected from the Illinois and Mississippi rivers. Sampling methodologies were developed to collect bacteria associated with various mussel organs and tissues, and the bacterial flora were characterized. This characterization of the bacterial flora of freshwater mussels provides a baseline for comparisons should mussel die-offs resume in the future.

17. Document Analysis a. Descriptors
Mussels, Aquatic animals, Illinois river, Mississippi river

b. Identifiers/Open-Ended Terms
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